Photoprotective Potential of Strawberry (Fragaria × ananassa) Extract against UV-A Irradiation Damage on Human Fibroblasts

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ABSTRACT: Exposure to UV-A radiation is known to induce discrete lesions in DNA and the generation of free radicals that lead to a wide array of skin diseases. Strawberry (Fragaria × ananassa) contains several polyphenols with strong antioxidant and anti-inflammatory activities. Because the major representative components of strawberry are anthocyanins, these may significantly contribute to its properties. To test this hypothesis, methanolic extracts from the Sveva cultivar were analyzed for anthocyanin content and for their ability to protect human dermal fibroblasts against UV-A radiation, as assayed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and Comet assays. Five anthocyanin pigments were identified using high-performance liquid chromatography–diode array detection–electrospray ionization/mass spectrometry. Moreover, the strawberry extract showed a photoprotective activity in fibroblasts exposed to UV-A radiation, increasing cellular viability, and diminishing DNA damage, as compared to control cells. Overall, our data show that strawberry contains compounds that confer photoprotective activity in human cell lines and may protect skin against the adverse effects of UV-A radiation.

KEYWORDS: strawberry, anthocyanin, antioxidants, DNA damage, fibroblast

INTRODUCTION: The exposure of skin to environmental insults such as smoke, microorganisms, or UV radiation can induce biological responses, including the development of hyperplasia, erythema, photoaging, and skin cancer.¹⁻³ In particular, UV-A radiation is able to penetrate through the dermis to subcutaneous tissue and may affect both epidermal and dermal skin components.⁴ At the cellular level, this component of the solar UV spectrum may cause oxidative damage through the generation of reactive oxygen species (ROS), which can interact with cellular biomolecules such as nucleic acids, proteins, fatty acids, and saccharides,⁵ altering the redox status of the intracellular milieu.

To mitigate such damage, the skin possesses extremely efficient defense mechanisms, including antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) and nonenzymatic antioxidant molecules (vitamin C, vitamin E, glutathione, and ubiquinone). However, because of constant environmental exposure to physical and chemical agents, an oxidant/antioxidant imbalance results that may have a profound effect on well-being. Topical or systemic treatment of skin with products containing antioxidant components could be a useful strategy for the prevention of UV-mediated cutaneous damages.⁶ Many studies have shown the efficacy of naturally occurring botanical antioxidants such as green tea polyphenols, silymarin, curcumin, apigenin, and resveratrol against UV radiation-induced inflammation and cancer.⁷⁻¹⁰ Similarly, it has been reported that topical application of plant-derived chemicals, such as caffeine or (−)-epigallocatechin gallate, inhibits carcinogenesis and selectively increases apoptosis in UV-B-treated mouse skin.¹¹

Strawberry (Fragaria × ananassa Duch.) is an important dietary source of bioactive compounds, most of which are natural antioxidants that contribute to the high nutritional quality of the fruit. Its remarkable value has been correlated to the high content of vitamin C, folate, and, more recently, to the high levels of vitamin B and bioflavonoids, especially anthocyanins, that are the quantitatively most important in strawberry.¹²⁻¹⁴ More than 25 different anthocyanin pigments have been described in strawberry fruits from different varietes.¹⁵ The major representative compounds show pelargonidin (Pg) as aglycone, although also some cyanidin (Cy) derivatives are generally observed. The presence of the two main anthocyanins (i.e., Pg-3-glc and in smaller proportion Cy-3-glc) seems constant in all varietes, but a qualitative and quantitative variability among cultivars is generally observed.

They possess strong antiinflammatory, antioxidant, antimuta-
gen, anticarcinogenic, and photoprotective properties and are...
able to modulate enzymatic pathways, thus, they play a role in preventing human diseases related to oxidative stress. The aims of this study were to characterize the strawberry anthocyanin content and to evaluate the possible protective effect of strawberry extracts on UV-A-induced skin damage using human dermal fibroblasts.

**MATERIALS AND METHODS**

**Strawberry Materials and Chemicals.** The strawberry cultivar Sveva was selected for the study. Ripe fruits were harvested from plants grown in an open experimental field for strawberry breeding and germplasm collection at the Azienda Agraria Didattico Sperimentale “P. Rosati” in Agugliano (Ancona, Italy). Within 2 h after harvest, whole fruits were stored at −20 °C prior to analysis.

2,4,6-Tripyridyl-s-trizine (TPTZ) was purchased from Fluka Chemie (Buchs, Switzerland), and sodium chloride (NaCl) and agarose were from Fisher Scientific, while all other reagents and solvents were from Sigma-Aldrich Chemical Co. (Milan, Italy). Minisart filters were obtained from PBI International, while AQUA C 18 150 mm × 4.6 mm columns were obtained from Phenomenex (Torrance, CA). Essential modified Eagle’s medium (EMEM) was purchased from Cambrex, as well as fetal bovine serum (FBS), glutamine, and antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin).

**Sample Preparation and Anthocyanin Extraction.** Frozen strawberries were thawed for 60 min at 4 °C; then, 10 g of the fruits was added to 100 mL (1:10 w/v) of extraction solution consisting of an 80% methanol solution acidified with 0.1% formic acid. Fruits were homogenized using an Ultraturrax T25 homogenizer (Janke & Kunkel, IKa Labortechnik) at 12000 rpm for 2 min, and the extraction was maximized by stirring the suspension for 2 h at 4 °C in the dark. The tubes were then centrifuged at 12000 g for 15 min (twice sequentially) to sediment solids, and supernatants were filtered through a 0.45 μm Minisart filter (PBI International), transferred to 5 mL amber glass vials, and then stored at −20 °C prior to analysis.

Anthocyanin extraction for high-performance liquid chromatography—mass spectrometry (HPLC-MS) analysis was performed as previously described. Frozen strawberries (50 g) were homogenized in methanol containing 0.1% HCl, kept overnight (~14 h) at 3−5 °C, and then filtered through a funnel under vacuum. The solid residue was exhaustively washed with methanol (4−6 times). The filtrates obtained were centrifuged (4000g, 15 min, 21 °C), and the solid residue was further subjected to the same process repeated until there was complete extraction of color. The aqueous extract obtained was washed with n-hexane to remove liposoluble substances, and then, 2 mL of the aqueous phase was carefully deposited onto a C-18 SepPaks Vac 6 cm3 cartridge (Waters). Sugars and more polar substances were removed by rinsing with 15 mL of ultrapure water and anthocyanin pigments eluted with 5 mL of methanol:0.1% trifluoroacetic acid (95:5). The methanolic extract was concentrated under vacuum in a rotary evaporator at −30 °C. Water was added to the extract to 2 mL and filtered through a 0.45 mm membrane filter (PBI international) prior to HPLC analysis.

**HPLC—Diode Array Detection (DAD)/Electrospray Ionization (ESI)-MS Analysis of the Anthocyanin Composition of the Extract.** Analyses were performed in a Hewlett-Packard 1100 series liquid chromatography. Separation was achieved on a 5 μm AQUA C 18 150 mm × 4.6 mm column (Phenomenex, Torrance, CA) thermostatted at 35 °C. Solvents used were (A) 0.1% trifluoroacetic acid in water and (B) HPLC-grade acetonitrile, establishing the following gradient: isocratic 10% B for 5 min, 10−15% B over 15 min, isocratic 15% B for 5 min, 15−18% B over 5 min, and 18−35% B over 20 min, using a flow rate of 0.5 mL min−1. Double online detection was carried out in a diode array spectrophotometer (DAS), using 520 nm as the selected wavelength, and a mass spectrometer (MS) connected to the HPLC system via the DAS cell outlet.

The mass spectrometer was a API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (50 psig) and turbo gas for solvent drying (600 °C, 40 psig). Nitrogen served as the curtain (10 psig) and collision gas (medium). The quadrupols were set at unit resolution. The ion spray voltage was set at 5000 V in the positive mode. Enhanced MS (EMS) was employed to detect all ions. Settings used were as follows: declustering potential (DP), 41 V; entrance potential (EP), 7.5 V; and collision energy (CE), 10 V. Enhanced product ion (EPI) mode was further performed to obtain the fragmentation pattern of the parent ion(s) in the previous experiment using the following parameters: DP, 41 V; EP, 7.5 V; CE, 25 V; and collision energy spread (CES), 0 V.

Anthocyanins were quantified from the areas of chromatographic peaks recorded at 520 nm compared to calibration curves obtained with external standards of Cy-3-glucoside (for Cy-based anthocyanins) and of Pg 3-glucoside (for Pg-based anthocyanins). Strawberry extracts were analyzes in triplicate.

**Culture of Human Dermal Cell (HuDe) Line and Cells Treatment.** Primary cell cultures of HuDe were purchased from the Central Laboratory of Istituto Zooprofilattico Sperimentale (Brescia, Italy). Fibroblasts were cultured in 25 cm2 flasks in EMEM (Cambrex) at 37 °C in a humidified atmosphere containing 5% CO2. Cells were seeded at a density of 0.5 × 104 cell/cm2, and the medium was changed every 2−3 days. At confluence, fibroblasts were dissociated by trypsinization with a solution containing 0.5 mg/mL trypsin and 0.2 mg/mL EDTA and then placed in 96-well plates 24 h prior to the experiment at a density of 2 × 103 cells/mL. Cells adhere to the 6 mm diameter well and reached over 75% confluence after 24 h.

To evaluate the potential cytotoxicity of the strawberry extracts, cells were incubated with different concentrations of strawberry extract, and cell viability was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The MTT assay is based on the reduction of a tetrazolium salt, (MTT) by intracellular dehydrogenases of viable living cells, leading to the formation of purple formazan crystals. According to the results obtained by this test, strawberry extracts were serially diluted in EMEM to achieve three different final concentrations (0.05, 0.25, and 0.50 mg/mL of extract). The methanol concentration was always below 1% of the final volume. Treated cells were incubated for another 24 h with the three distinct concentrations of Sveva extracts, while the same quantity of methanol administered to the treated cells was added to control cells. After incubation with strawberry extracts, cells were exposed to UV-A light (see below).

**Treatment with UV-A Light.** Control and pretreated cells were washed twice with phosphate-buffered saline (PBS) and covered with a thin layer of PBS prior to exposure. The 96-well plate was then covered with the 2 mm thick quartz slab on which a dark cardboard sheet was attached. The cells were then placed on a brass block embedded on ice to reduce the temperature and hence the evaporation during exposure, which could eventually dry out the medium. The UV-A irradiating source was a Phillips Original Home Solarium (model HB 406/A; Philips, Groningen, Holland) commercial sun lamp equipped with a 400 w ozone-free Philips HPA lamp, UV type 3. The source delivered 23 mW/cm2 between 300 and 400 nm at a distance of 20 cm from the cell cultures. It was always prerun for 10 min to allow the output to stabilize. The incident dose of UV-A received by the samples was 275 kJ/m2, that is, the dose approximately equivalent to about 90 min of sunshine at the French Riviera (Nice) in summer at noon. Five 104 cells/cm2 were exposed to UV-A light (see below).

**Evaluation of Protection Against UV-A-Induced Damage.** Cells Viability. After UV-A exposure, control and pretreated cells were washed twice with PBS and incubated with a salt solution of MTT at a concentration of 0.5 mg/mL for 2 h at 37 °C. Then, the medium was removed, and the crystals were dissolved in DMSO. The optical density of the suspension was measured at 595 nm using a microplate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA).

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was read at 550 nm using a microplate reader (Synergy HT, Biotek, Winooski, VT). Cell viability was expressed as a percentage of live cells as compared to the control. The data reported represent average values from at least three independent experiments.

**Evaluation of DNA Damage by the Comet Assay.** The comet assay was performed as described by Singh et al. with modifications by Tice et al. The comet assay is a sensitive technique that allows visualization of DNA damage, essentially by electrophoresing the DNA from single cells from immobilized nuclei. Smaller fragments migrate faster, so highly damaged cells will produce “comets” that may be measured with ethidium bromide staining and fluorescent microscopy. Briefly, control and pretreated cells cultured in 12-well plates were washed twice with PBS and exposed to UV-A in 2 mL of PBS as described above for 12 min. PBS was then removed, cells were detached by trypsinization, EMEM was added, and cells were counted in a Kova Glastic Slide 10 with grid chamber. Aliquots containing ~50 000 cells from each sample were transferred to Eppendorf tubes and centrifuged for 15 min at 1200 rpm at 4 °C. The supernatant was removed, and cells were resuspended in 110 μL of 1% low melting agarose. Fifty microliters was then rapidly placed on a 1% agarose gel containing 1% Triton X-100. The slide was then stained by adding 100 μL of ethidium bromide (1 μM) to allow speculation about their identity.

**RESULTS AND DISCUSSION**

**Anthocyanin Composition Determined by HPLC-DAD-ESI-MS/MS.** Anthocyanins in strawberry are the best known polyphenolic compounds and quantitatively the most important. In the present work, five anthocyanin pigments were detected according to their UV-vis mass spectral characteristics. The HPLC anthocyanin profile of Sveva cultivar, obtained in positive mode analyses (retention time in the HPLC system, λmax in the visible region, molecular ion, main fragments observed in MS2, and total content) is summarized in Table 1. Most of the compounds identified are derived from Pg. In addition to the compounds indicated in the table, other very minor pigments were also detected, although no good absorption or mass spectra could be obtained to allow speculation about their identity.

### Table 1. Retention Times (Rt), Wavelengths of Maximum Absorption in the Visible Region (λmax), Mass Spectral Data, Tentative Identification, and Quantification of Anthocyanin Pigments in Strawberry Sveva

<table>
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<tr>
<th>Peaks</th>
<th>Rt (min)</th>
<th>λmax (min)</th>
<th>Molecular Ion [M+H]+</th>
<th>MS2</th>
<th>Tentative Identification</th>
<th>Quantification (mg/kg)</th>
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<tr>
<td>1</td>
<td>23.2</td>
<td>515</td>
<td>449</td>
<td>287</td>
<td>Cy-3-glucoside</td>
<td>16.35</td>
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<tr>
<td>2</td>
<td>27.3</td>
<td>502</td>
<td>433</td>
<td>271</td>
<td>Pg-3-glucoside</td>
<td>611.18</td>
</tr>
<tr>
<td>3</td>
<td>29.3</td>
<td>503</td>
<td>579</td>
<td>433, 271</td>
<td>Pg-3-rutinoside</td>
<td>1.86</td>
</tr>
<tr>
<td>4</td>
<td>37.7</td>
<td>504</td>
<td>519</td>
<td>271</td>
<td>Pg-3-malonyglucoside</td>
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<tr>
<td>5</td>
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<td>271</td>
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<td>Total Content</td>
<td></td>
<td></td>
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</tbody>
</table>

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Statistical Analysis. All results are expressed as means ± standard errors of the mean (SEMs). Statistical analysis was done using the one-way analysis of variance and Tukey’s posthoc test; p ≤ 0.05 was considered significant.
with antioxidant properties. In recent years, natural compounds such as vitamins and phenols have gained considerable attention as protective agents, which could be added in preparations for topical applications. Fruits and vegetables are rich in diverse nutrients, which can act as chemoprotectants. These structurally different components possess complementary and overlapping potential protective actions, including antioxidant and anti-inflammatory properties, as well as enhanced activity and expression of detoxification enzymes and strengthened immune system health.26

Various components of plant and fruit have been investigated for their potential protective capacity. Strawberry has attracted significant attention in recent years on micronutrient and beneficial phytochemicals contents. The major class of phenolic compounds in strawberry is represented by flavonoids (mainly anthocyanins, with flavonols and flavanols giving a minor contribution), followed by hydrolyzable tannins (ellagitannins and gallotannins) as the second most abundant class, and phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids) together with condensed tannins (proanthocyanidins), being the minor constituents. Ellagitannins (ETs) are together with anthocyanins the most abundant phenolic compounds in strawberry.13 They are different combinations of hexahydroxydiphenic acid with glucose, with a wide range of structures such as monomers (i.e., ellagic acid glycosides), oligomers (i.e., sanguin H-6, the most typical ET in strawberry), and complex polymers. Together with gallotannins, they are also called hydrolyzable tannins and, upon hydrolysis, release ellagic acid, although other metabolites can also be produced and are distinctive of individual ETs (i.e., gallic acid). The ETs content and composition in foodstufs has been characterized only recently so that more studies are desirable in this field because of their important impact on human health. For example, ellagic acid is the phenolic component primarily associated with the chemopreventive effects, appearing to function as an anticarcinogen at the initiation and postinitiation stages of tumor development in in vitro and in vivo experiments.27 Interestingly, in the past decade, the antitumorigenic properties of the distinct classes of phenolic components have been also demonstrated. In particular, the anthocyanin-rich25,28 and tannin-rich fractions of different berry extracts, the latter mainly including ellagitannins29 or proanthocyanidins30 depending on the berry species, appear to contain key anticarcinogenic components of berries against multiple human cancer cell types in vitro and in vivo animal model tumor systems.

Although their presence is minor in strawberry from a quantitative point of view, a relevant interest is focused on flavonoids. They are found in strawberry in both monomeric (catechins) and polymeric form called condensed tannins or procyanidins, especially in flesh and achenes.12 They have been reported to directly and indirectly possess antioxidant, antimicrobial, antiallergic, antihypertensive, and inhibition of the activities of some physiological enzyme and receptors.35

Finally, strawberries are important fruits also because of their extremely high content of vitamin C, even higher than citrus fruit, which makes them an important source of this vitamin for human nutrition. Vitamin C is an essential compound, with several biological functions in humans. In fact, the results of numerous prospective, observational as well as case-control studies, indicate that higher intakes of vitamin C from diet, from diet plus supplements, or from supplements use itself are often correlated with a lower incidence of cardio and cerebrovascular diseases,36 most types of cancers,37 and other health problems such as lead toxicity. In addition to disease prevention, vitamin C supplementation seems to be a beneficial adjunct to conventional therapies for individuals with atherosclerosis,38 hypertension, diabetes mellitus, and several types of cancers.

In the present work, we studied the potential photoprotective capacity of strawberry cultivar Sveva extract against UV-A damage on human fibroblasts. First, the possible toxic effect of the extracts in relation to their increasing concentration and exposure time was studied. Cell vitality did not vary with increases in strawberry extract concentration or the exposure time, thereby demonstrating that the extracts were not cytotoxic under the experimental conditions (data not shown). Next, control cells were subjected to various fluences of UV-A and immediately analyzed by MTT. For times more than 30 min, the treatment caused a significant loss of viability (Figure 1). Using this range of fluences, control cells and cells pretreated with different concentrations of strawberry extract (0.05, 0.25, and 0.5 mg/mL) were exposed and analyzed with MTT to determine the possible photoprotective activity of Sveva extracts and if it was dose-dependent. Cells preincubated with the lower concentration of the extract exhibited vitality similar to the control, while the cells preincubated with higher concentrations of the extract showed higher survivability. In particular, cells treated with 0.5 mg/mL exhibited a significant difference in vitality (p < 0.05), especially for exposure between 5 and 15 min (Figure 2). However, at higher fluence rates, the differences were not significant. This outcome is likely due to the depletion of the protective capacity of strawberry extracts after high UV treatments. Moreover, to assess whether Sveva extract could impart DNA protection, control cells and cells preincubated with strawberry were exposed to UV-A for 15 min and processed according the Comet Assay protocol.20,21 Consistent with the vitality assay, DNA damage was observed in control cells and cells preincubated with the lower concentrations of the extract (0.05 mg/mL). Conversely, cells pretreated with 0.25 and 0.5 mg/mL of extracts showed a highly significant decrease (p < 0.05) in DNA damage in comparison with the control (Figure 3).

Our results indicate that strawberry extracts provide photoprotective effects to dermal cells in vitro. It is exciting to speculate that the same compounds may be helpful in
Youdim and co-workers\textsuperscript{43} reported that anthocyanins are incorporated into the membrane and cytosol of endothelial cells and significantly enhance their resistance to the damaging effects of several chemical oxidative stressors. Finally, Tarozzi and co-workers showed that C3G reduced DNA damage induced by H$_2$O$_2$ and O$_2$ in HaCaT cells.\textsuperscript{41}

This work provides a basis for more in-depth studies to examine the efficacy of strawberry fruit in the prevention of UV-induced stress. Future studies will examine the bioavailability of these molecules at dermal level when they are introduced through topical application.

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\subsection*{Notes}
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\section*{ABBREVIATIONS USED}

HuDe, human dermal cells; EMEM, essential modified Eagle medium; ROS, reactive oxygen species; Cy, cyanidin; Pg, pelargonidin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

\section*{REFERENCES}


